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Research Article

RESEARCH ARTICLE

Bacterial density rather than diversity correlates with hatching success across different avian species

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One sentence summary: Hatching success is predicted by bacterial density in general and by the bacterial community assemblage on eggshells when minority bacterial species are included. Interestingly, hatching success is similar in different species, suggesting interspecific differences in antibacterial defenses.

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ABSTRACT

Bacterial communities within avian nests are considered an important determinant of egg viability, potentially selecting for traits that confer embryos with protection against trans-shell infection. A high bacterial density on the eggshell increases hatching failure, whether this effect could be due to changes in bacterial community or just a general increase in bacterial density. We explored this idea using intra- and interspecific comparisons of the relationship between hatching success and eggshell bacteria characterized by culture and molecular techniques (fingerprinting and high-throughput sequencing). We collected information for 152 nests belonging to 17 bird species. Hatching failures occurred more frequently in nests with higher density of aerobic mesophilic bacteria on their eggshells. Bacterial community was also related to hatching success,

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but only when minority bacterial operational taxonomic units were considered. These findings support the hypothesis that bacterial density is a selective agent of embryo viability, and hence a proxy of hatching failure only within species. Although different avian species hold different bacterial densities or assemblages on their eggs, the association between bacteria and hatching success was similar for different species. This result suggests that interspecific differences in antibacterial defenses are responsible for keeping the hatching success at similar levels in different species.

Keywords: ARISA; avian community; bacterial community; bacterial density; comparative analysis; eggshells; hatching success; high-throughput sequencing; Illumina HiSeq; Phylogenetic General Least Square

INTRODUCTION

Bacteria and other microorganisms are a key component of the environment where animals develop and reproduce and, hence, play a central role in the evolution of life history and antimicrobial defensive traits of animals (McFall-Ngai *et al.* 2013). For instance, microorganisms have an important role in hatching success, and hence, avian fitness in natural conditions (Cook *et al.* 2005a, 2005b). Avian eggs are suitable environments for the growth of opportunistic and potential pathogenic bacteria, not only because of their high nutrient content, but also because the temperature necessary for egg incubation is close to the optimal temperature for the exponential growth of these bacteria (Krieg and Holt 1984; Board and Fuller 1994; Singleton and Harper 1998). Bacterial pathogens infect the embryo through the eggshell pores, trans-shell bacterial infection thus requires that bacteria first colonize the eggshell and multiply (Board and Tranter 1986). Bacteria that can potentially act as pathogens of avian embryos are ubiquitous and have been considered an important evolutionary force selecting for different physical, chemical and behavioral barriers that impede bacterial trans-shell colonization and growth inside the eggs (Board *et al.* 1994; Deeming 2002; Wellman-Labadie, Picman and Hincke 2007).

Avian incubation reduces bacterial density and provokes changes in bacterial assemblages on eggshells, thereby lowering the risk of embryo and egg content infection (Cook *et al.* 2005a, 2005b; Shawkey *et al.* 2009). The role of incubation in bacterial shifts on the eggshells has been corroborated in subsequent studies (Ruiz-de-Castañeda *et al.* 2011b; Ruiz-De-Castañeda *et al.* 2012; Potter *et al.* 2013; Brandl *et al.* 2014; Grizard *et al.* 2014; Lee *et al.* 2014; Grizard *et al.* 2015). These studies suggest that high bacterial densities should be correlated with poor hatching, and will reduce avian fitness overall. However, negative correlation between bacterial densities and hatching success of avian eggs in natural conditions is limited to a few studies, while many others found no association. Hansen *et al.* (2015) found a negative correlation between eggshell bacteria and hatching success within populations of greater white-fronted geese, as did a comparative analysis of hatching success and eggshell bacterial loads from two European bird populations (Soler *et al.* 2012). Other studies found no association between bacteria on the eggshell and hatching success. For instance, incubation reduced bacterial density on eggshells in the pied flycatcher (*Ficedula hypoleuca*) (Ruiz-De-Castañeda *et al.* 2012), but this reduction in bacterial density did not covary with hatching success (Ruiz-de-Castañeda *et al.* 2011a). Similarly, no association was detected in four other Mediterranean species (Peralta-Sánchez *et al.* 2010; Wang, Firestone and Beissinger 2011).

Variation in the relationship between eggshell bacteria and hatching success may be related to a range of factors that influence the diversity and abundance of eggshell bacteria, including geography, differences in nest construction and differences in antimicrobial behaviors across bird species. For instance, studies showing that growth of eggshell bacterial communities differ for birds in Mediterranean and tropical areas highlight the importance of considering geographic variation (Cook *et al.* 2005b; Wang, Firestone and Beissinger 2011). Moreover, we also know that environmental conditions in avian nests with different structural characteristics (i.e. orientation, open *vs.* hole nests) affect bacterial communities of the eggshells (Goodenough and Stallwood 2012; Peralta-Sánchez *et al.* 2012), which therefore may alter the association with hatching success. The relationship between characteristics of eggshell bacterial communities and trans-shell bacterial infection or hatching failures would depend on antimicrobial properties of eggshells, which can vary both intra- and interspecifically (Wellman-Labadie, Picman and Hincke 2007, 2008b; Horrocks *et al.* 2014; Martín-Vivaldi *et al.* 2014; D'Alba *et al.* 2016). Thus, detecting associations between the bacterial environment and infection (or hatching failure as its consequence) will probably vary according to environmental conditions and species-specific antimicrobial capabilities.

In the present study, we tested the hypothesis that bacteria on eggshells negatively impact hatching success. We explore correlative prediction of this hypothesis by studying bacterial communities of eggshells in an avian community, and exploring the association with hatching success both within and between species while controlling for potentially temporal and geographical confounding effects (i.e. using a single year and location). Bacterial communities were characterized both by traditional culture methods and molecular methodologies. We hypothesize that both bacterial densities and assemblages would be related with hatching success at the intra and the interspecific levels, predicting that nests and species that experience higher bacterial density or a more harmful bacterial assemblage will experience higher hatching failures. However, we cannot dismiss other alternative predictions as a lack of interspecific covariation between hatching success and eggshell bacterial when antimicrobial defenses are on average pretty adjusted to the strength of selection pressure at the species levels. Finally, the use of two different culture independent techniques allows us to study their ability to detect the association between hatching success and bacterial community. We hope that this study will provide new pieces of evidence for the relationships between microorganisms and birds, and help to better understand the selective pressures acting at the nest environment.

MATERIAL AND METHODS

Study system

The study area was located in the Hoya de Guadix (37°18'N, 3°11'W), a high-elevation plateau, 1000 m above sea level, with a semi-arid climate. During the breeding season of 2007 (March–July), 600 nest-boxes were checked once per week, and an intense search of other nests of wild birds (including hole- and open-nesters) was performed. Nest-boxes were cork-made

(height × width × depth: 370 × 200 × 230 mm, bottom-to-hole height: 250 mm, hole diameter: 60 mm, height from ground around 2 m). Once a new nest was found, it was visited every 2–3 days to determine laying date and clutch size. In order to assess hatching success accurately, we visited each nest every day from 2–3 days before the estimated hatching date of the first egg until 2–3 days after the end of hatching. This parameter was estimated as the proportion of eggs detected in the nests 2–3 days before hatching (i.e. after possible partial predation events) that successfully hatched in non-predated clutches. Only data from clutches where at least one egg hatched were included in the analyses, in order to avoid possible bias as it is to consider unfertile clutches or nests that experience partial abandonment undetected by us.

We got data from 152 clutches from nests belonging to 17 species (Table 1).

Bacterial sampling and culturing

Bacteria from the eggshells were sampled at the beginning of the incubation, a couple of days after clutch was completed. In order to avoid contamination between nests, all samples were collected wearing latex gloves washed with 96% ethanol. Eggs were sampled by gently cleaning their shells with a sterile swab slightly wet with sterile 0.2 M sodium phosphate buffer (pH = 7.2). All eggshells from the same clutch were sampled with the same sterile swab. Swabs were preserved in the same phosphate solution in a microcentrifuge tube (1.2 mL) that was stored in a cooler at 4–6°C. Once in the laboratory, samples were stored at 4°C until processing (range: 0–45 days). After bacterial sampling, length and width of all eggs were measured with a caliper (accuracy 0.02 mm) to estimate sampled surface of eggshell.

Microorganisms were cultured by homogenously spreading 100 μL of the suspensions at serial dilutions on plates with Tryptic Soy Agar (Scharlau Chemie S.A. Barcelona), a broadly used general medium to grow total aerobic and mesophilic bacteria. Agar plates were incubated at 32°C for 72 h, and the swab and remains of each sample were frozen at −20°C for subsequent molecular analyses. Eggshell bacterial density was estimated as the number of Colony Forming Units that grew in Tryptic Soy Agar per egg surface unit (cm^2) for all eggs sampled following Peralta-Sánchez et al. (2012). Detailed information is described in Electronic Supplementary Material 1 (ESM 1).

DNA extraction

DNA extractions were performed by Chelex-based extraction protocol following Martín-Platero et al. (2010). DNA templates were amplified and analyzed by means of automatic ribosomal intergenic spacer analysis (ARISA) and high-throughput sequencing (HiSeq) Illumina. Detailed information is described in ESM 1.

Automatic ribosomal intergenic spacer analysis (ARISA)

Polymerase Chain Reaction amplification of the 16S-23S intergenic spacer region in the rRNA operon was performed with a fluorescence-labeled forward primer (72F: 5′-TGC GGC TGG ATC TCC TT-3′, labeled with the phosphoramidite dye 5-FAM; 38R: 5′-CCG GGT TTC CCC ATT CGG-3′) (Ranjard et al. 2000). PCR reactions were performed at a final volume of 50 μl and the reaction mix contained 1x PCR buffer (75 mM Tris HCl, pH 9.0; 50 mM KCl; 20 mM $(NH4)_2SO_4$), 2.5 mM Cl_2Mg, 200 μM dNTPs, 0.1 μM of each primer, 1 U Taq DNA polymerase (EMBL, Spain) and 10 ng DNA template. PCR started with a denaturing step at 94°C for 2 min; followed by 30 cycles of amplification of denaturing at 94°C for 1 min, an annealing step at 55°C for 30s and an extension step at 72°C for 1 min; and a final elongation step of 72° C at 5 min. PCRs were performed in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). The PCR products were checked on 0.9% agarose gels. Determination of amplicon size was performed in the Sequencing Services of the University of Granada using a genetic sequencer ABI Prism 310 (Genetic Analyzer, Applied Biosystems, USA).

Amplicon profiles were determined with Peak Scanner 1.0 (Applied Biosystems, USA) to set operational taxonomic units (OTUs). *Automaticbinner.r* and *interactivebinner.r* scripts (Ramette 2009) in R environment (R Core Team 2015) (range 100–1200 base pairs; min RFI = 0.09; window width = 4; shift = 0.1) were used to obtain a table of 227 OTUs (Table 1). Because of the inaccuracy of using peak areas in ARISA as indicative of OTUs' abundance, we used the conservative approach of using presence–absence information to build the OTU table. A total of 190 OTUs were used in subsequent analyses. This OTU table comprised information of the bacterial community of each sampled nest (intraspecific approach). Afterwards, this OTU table was used to estimate prevalence of each OTU for each bird species that was used in the interspecific approach.

High-throughput sequencing

16S rRNA variable region 4 (V4) were sequenced in Illumina HiSeq 2000 Platform following the protocols of the Earth Microbiome Project (Gilbert et al. 2010; Thompson et al. 2017). After amplification, primers were trimmed, sequences were demultiplexed and quality filtering was performed, following QIIME software v1.9 (Quantitative Insights In Microbial Ecology; Caporaso et al. 2010). Sequences are available in QIITA repository (https://qiita.ucsd.edu/; study ID: 1632). QIIME is a wrapper software that allows to perform both upstream (from the raw sequences files to the OTU table) and downstream analyses (from the OTU table to final publishing results) in a single pipeline (Navas-Molina et al. 2013). Open reference OTU picking procedure (Rideout et al. 2014) was applied to generate the OTU table, clustering sequences against Greengenes database v10_13 (DeSantis et al. 2006; McDonald et al. 2012). Subsequently, Archaea, chloroplast, mitochondria and non-phylum assigned OTUs were filtered as well as singletons and OTUs with frequency lower than 0.005% of the total sequence account (Bokulich et al. 2013). In order to control for the sequencing effort, the OTU table was rarefied at 10 569 sequences, and a total of 548 OTUs were retained for subsequent analyses (Table 1). More detailed information on this procedure can be found in the ESM1. This OTU table contained information about the bacterial community of each sampled nest (intraspecific approach). Afterwards, this OTU table was used to estimate prevalence of each OTU for each bird species that was used in the interspecific approach.

Sample size and statistical analyses

Intraspecific approach

This approach studied the association between hatching success and bacteria community on eggshells (density and assemblage) at different bird nests after controlling for the species identity in the statistical models.

Bacterial density and OTU richness in ARISA were normally distributed after log_{10}-transformation (Kolmogorov–Smirnov tests for continuous variables, $P > 0.05$). OTU richness in HiSeq

Table 1. Values of arcsin of hatching success (percentage of hatched eggs from the clutch), log-transformed values of the geometric means of bacterial density on the eggshells (log10-tranformed number of colonies corrected for eggshell surface and dilution titer that grew in media for mesophilic bacteria) and OTU richness in ARISA (automated ribosomal intergenic spacer analysis) and Hiseq (high-thoughput sequencing using HiSeq1000 Illumina platform).

		Cultures			ARISA		HiSeq	
Bird species	Hatching success	N[a]	Log10 density	Density (CFUs/cm^2)	N[a]	OTU richness	N[a]	OTU richness
Athene noctua	100.0%	8	6.773	5929 253.25	8	87	8	308
Columba livia	99.2%	9	3.836	6854.88	8	90	8	371
Coracias garrulus	97.5%	10	6.287	1936 421.96	10	132	10	391
Corvus monedula	94.8%	8	5.961	914 113.24	6	61	8	382
Falco tinnunculus	100.0%	3	7.501	31 695 674.63	3	39	3	303
Hirundo rustica	95.0%	9	5.849	706 317.55	8	84	9	349
Lanius meridionalis	91.2%	4	4.996	99 083.19	4	16	4	337
Oenanthe leucura	93.7%	6	6.644	4405 548.64	5	65	5	264
Otus scops	98.6%	9	5.291	195 433.95	9	128	9	384
Parus major	96.4%	10	6.602	3999 447.50	9	82	10	386
Passer montanus	95.2%	7	6.085	1216 186.00	7	63	7	330
Pica pica	92.0%	15	1.128	13.43	2	11	5	306
Pyrrhocorax pyrrhocorax	90.4%	4	5.428	267 916.83	4	39	4	271
Serinus serinus	95.7%	5	4.913	81 846.48	5	70	4	292
Sturnus unicolor	91.3%	13	5.799	629 506.18	12	105	13	434
Turdus merula	89.2%	19	5.662	459 198.01	17	112	19	469
Upupa epops	82.3%	13	6.625	4216 965.03	8	80	10	404

[a]Sample sizes (N) used in different analyses are also reported.

was normally distributed without transformation. Moreover, the distribution of residuals of arcsine-transformed hatching success in all tested models did not differ from normality (Kolmogorov–Smirnov tests for continuous variables, $P > 0.05$), so we were confident in the use of parametric statistical tests with the transformed variables. The predicted influence of bacterial density or OTU richness on hatching success was tested by means of general lineal mixed models (GLMM) with hatching success as the dependent variable, species identities as an independent random factor (random intercept models) and bacterial density or OTU richness as a covariate. We also tested whether the predicted effect of bacterial density differed for different species by estimating the effect of the interaction between species identity (random factor) and bacterial density. Significant interactions indicate that the slopes associated to the relationship between hatching success and bacterial density differ for different species, while non-significant effects suggest similar effects independently of the species identity. All analyses used two-tailed *P*-values throughout, and were conducted using STATISTICA 10 software.

Procrustes ANOVA was used to explore the relationship between hatching success and bacterial assemblage. This permutational statistical test is equivalent to the classical ANOVA test, but in a multivariate setting. First, the Gower distance matrix was calculated from the ARISA OTU table as recommended for binary data (presence/absence) by Kuczynski *et al.* (2010), and Weighted and Unweighted UniFrac distance matrix from Hiseq OTU table (Lozupone and Knight 2005). UniFrac distance is a measure of the distance between communities based on their phylogenetic structure and is recommended when phylogenetic information of the bacterial community is available (i.e. abundance OTU tables from high-throughput sequencing) (Lozupone and Knight 2005; Lozupone *et al.* 2011). Second, Principal Coordinates Analyses (PCoA) axes were calculated from distance matrixes. Third, we used the complete set of PCoA axes as explanatory matrix for the Procrustes ANOVA, that were performed in the R environment (R Core Team 2015) using the packages 'vegan' (Dixon 2003) and 'geomorph' (Adams and Otarola-Castillo 2013).

Finally, Spearman correlations were performed to explore the relationship between hatching success and OTU abundance only with the HiSeq approach at different taxonomic levels (Phylum, Class, Order and Family) (similar analysis using OTU richness from ARISA was not possible as we use presence-absence OTU table). False recovery rate corrections were applied to correct for multiple comparisons.

Interspecific approach

For each bird species, we calculated the average values of bacterial density and the prevalence of each OTU from the ARISA and HiSeq OTU tables. Specific information cannot be considered statistically independent due to common ancestry, so we considered phylogenetic relationships between species to perform comparative analyses (Harvey and Pagel 1991). We used Phylogenetic Generalized Least Square regression (PGLS) analyses (Pagel 1997, 1999). Implementation and use of the PGLS analyses is thoroughly explained in Møller *et al.* (2011) and Soler *et al.* (2011a). We weighted all species by sample size to avoid potential differences in sampling effort (Garamszegi and Møller 2010, 2011; Vincze *et al.* 2013).

Geometric mean values of each bacterial density and average hatching success for each bird species were calculated. Distribution frequencies of these variables did not differ from normality after $\log_{10}$- and arcsine transformations, respectively (Kolmogorov–Smirnov normality tests, $P > 0.15$). Bacterial density significantly varied between species (GLM, bacterial density as dependent variable, species identity as factor, $F_{16,125} = 1.83$, $P = 0.034$), which justified the use of mean values in our comparative analyses (Peralta-Sánchez *et al.* 2012).

Prevalence of each OTU in each bird species was calculated from ARISA and HiSeq OTU tables. Following similar steps as in the intraspecific approach, Gower distance matrix were calculated from ARISA OTU table, and Weighted and Unweighted UniFrac distance matrixes from HiSeq OTU table. PCoA were performed from these distance matrixes and the complete set of axes was used in the Procrustes ANOVA.

Phylogenetic relationships were based in Thuiller *et al.* (2011) and modifications in the phylogenetic tree were performed using TreeGraph2 free software 2.11.1-654 beta (Fig. 1).

Phylgenetic general least square (pGLS) models were performed in the R environment (R Core Team 2015). For bacterial density analyses, 'MASS' (Venables and Ripley 2002), 'ape' (Paradis, Claude and Strimmer 2004) and 'mvtnorm' (Genz *et al.* 2009) packages were used as well as an additional function by R. Freckleton (University of Sheffield) implemented in the package 'caic'. For bacterial assemblages analyses, 'vegan' (Dixon 2003) and 'geomorph' (Adams and Otarola-Castillo 2013) packages were used.

Relationship between bacterial density and bacterial community

At the intraspecific level, GLMM were performed comparing bacterial density and OTU richness and species as random factor and Procrustes ANOVA for comparing bacterial density and bacterial assemblages including species identity as factor (ARISA and HiSeq). Bacterial density correlated positively with bacterial species richness as well as with bacterial assemblages when consider HiSeq bacterial richness, but this was not the case for ARISA bacterial richness (ESM 2). For comparative analyses, a similar approach was performed: pGLS tested the association between bacterial density and OTU richness and bacterial assemblages. No significant relationship was found between those variables (ESM 2).

RESULTS

Microbiology of avian eggshells

Gammaproteobacteria (58.0% average abundance, phylum Proteobacteria) was the most dominant class in bacterial community on eggshells followed by Actinobacteria (18.1%, phylum Actinobacteria), Bacilli (11.9%, phylum Firmicutes) and Sphingobacteriia (5.6%, phylum Bacteroidetes). This dominant community is common among and within species (Fig. 1). However, Actinobacteria or Bacilli dominated over other classes in some communities as well as Sphingobacteriia did in only one clutch (Fig. 1). *Pseudomonadaceae* was one of the most dominant families in the bacterial communities on eggshells (94.1% of samples, average abundance 31.0%). Within *Pseudomonadaceae*, the genera *Pseudomonas* were the most dominant (94.1% of samples, average abundance 30.4%). The other two most dominant families were *Micrococcaceae* (96.3% of samples, average abundance 9.8%) and *Enterobacteriaceae* (92.6% of samples, average abundance 2.50%). *Arthrobacter* was the most dominant genera of the family *Micrococcaceae* (93.2% of samples, average abundance 5.60%), while one unidentified species was the most dominant of the family *Enterobacteriaceae* (94.85% of samples, average abundance 8.30%). Abundance of different taxa are shown in Fig. 1 and ESM 4.

Intraspecific variation in hatching success and in bacterial density and community

Hatching success was negatively correlated with the density of aerobic mesophilic bacteria after controlling for bird species identity (Table 2; Fig. 2). That effect of bacterial density on hatching success did not depend on species identity (GLMM, interaction species identity x bacterial density, $F_{17,118} = 0.67$, $P = 0.824$).

After controlling for bird species identity, hatching success was only significantly explained when using Unweighted UniFrac (Table 2; Fig. 3). Finally, none of the OTUs were correlated significant with hatching success at different taxonomic levels (ESM 3).

Interspecific variation in bacterial assemblages and hatching success

Bacterial assemblages on eggshells were very similar among bird species (Table 2; Fig. 4). Interspecific variation in eggshells bacterial density was not associated with differences in hatching success (Table 3; Fig. 4). Moreover, among species variation in bacterial assemblages was not significantly related with interspecific variation in hatching success with any of the molecular approaches (Table 3; Fig. 4).

DISCUSSION

The main goal of this manuscript was to explore the effects of bacterial community of the avian eggshells on the hatching success in a community of birds. Our main findings are that (i) variation in eggshell density of mesophilic bacteria is negatively related to within species variation in hatching success of wild birds and that (ii) this effect was similar for all species considered in our study. Moreover, (iii) Unweighted UniFrac distance between bacterial communities covaried with hatching success. At the interspecific level, neither (iv) eggshell bacterial densities, nor (iv) bacterial assemblages covaried with hatching success. These results suggest that bacteria on the eggshell influence reproductive success of birds, and that this effect is similar in different taxa.

Although a very low percentage of the bacterial diversity grow in mesophilic media in aerobic conditions (Amann, Ludwig and Schleifer 1995; Pace 1997), mesophilic bacterial counts have been used to quantify bacterial abundance (Cook *et al.* 2003; Cook *et al.* 2005a, 2005b). Moreover, conclusions achieved by culture-dependent estimations of bacterial abundance on eggshells have been validated by using molecular characterization (i.e. diversity) of communities (Spanggaard *et al.* 2000; Cook *et al.* 2005a; Shawkey *et al.* 2009). In addition to traditional culture techniques, we also employed two different molecular methods: ARISA and Hiseq. Both techniques differ in the rRNA regions (ARISA amplifies the ITS region; and in our case HiSeq amplified the V2-V4 region of 16s rRNA), their ability to detect OTUs (from 190 using ARISA to 548 using HiSeq) and, consequently, the possibility to identify different taxonomic groups influencing hatching success. ARISA provides a fingerprinting of the bacterial community while Hiseq returns the phylogenetic information of the OTUs (i.e. bacterial group identity) (Fisher and Triplett 1999; Corneo *et al.* 2013). Especially important is the comparison between ARISA and Unweighted Unifrac results as both approximations use OTU presence/absence information. We found a significant effect of the bacterial assemblage in hatching success only when Unweighted Unifrac was tested. Both approaches have been proposed as complementary (Lee *et al.* 2014), although in our study Hiseq has performed better than ARISA. Although

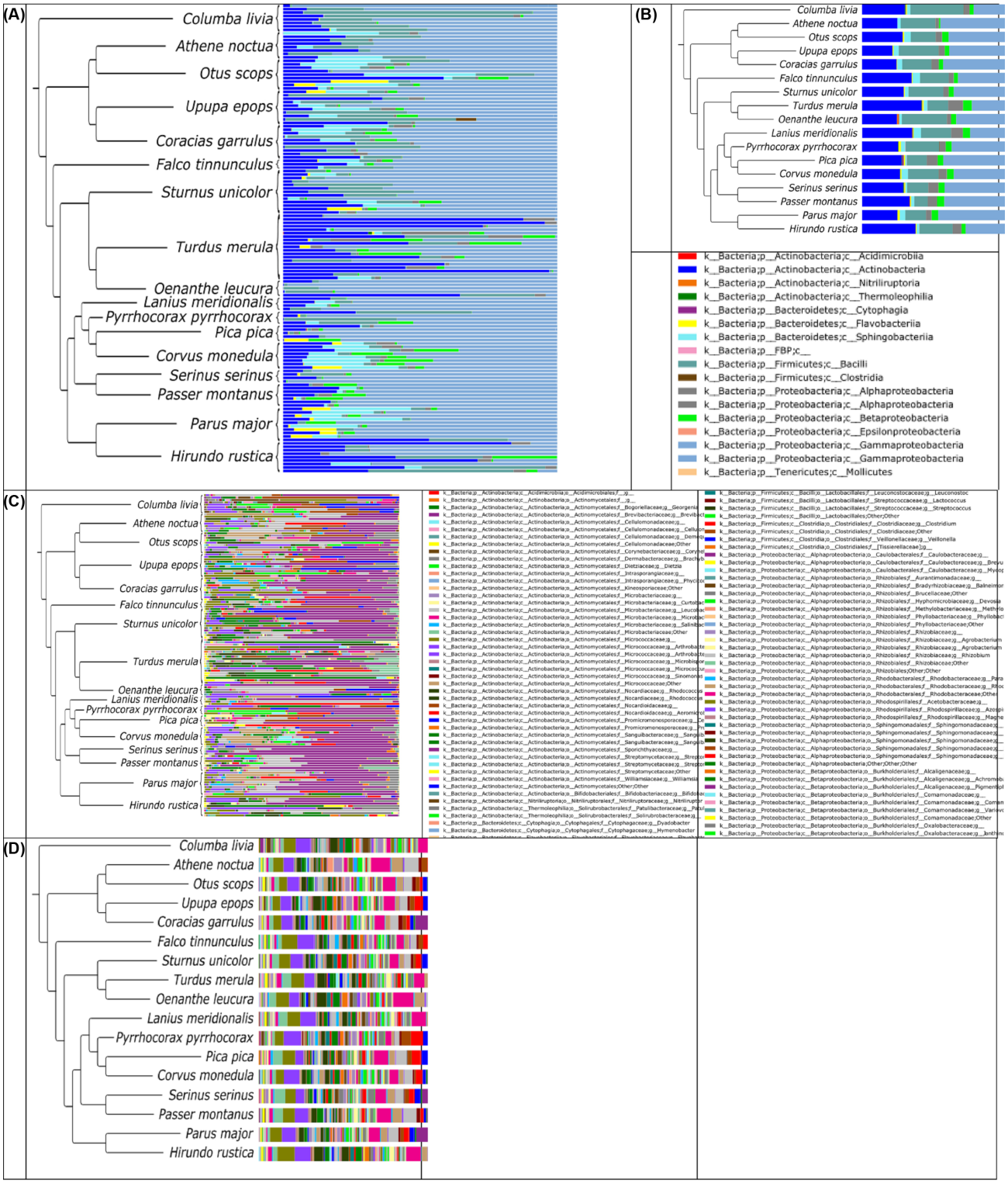

Figure 1. Summary plots representing prevalence of bacterial phyla and classes on bird eggshells for 17 avian species (A); prevalence of bacterial phyla on eggshells per bird nest (B); prevalence of bacterial Genera for 17 avian species (C); prevalence of bacterial for 152 clutches of 17 avian species (D). Avian phylogenetic tree was modified from Thuiller *et al.* (2011).

Table 2. Statistical analyses performed in order to explore the effects of bird species identity and bacterial density (log-transformed), OTU richness and bacterial assemblages on hatching success (arcsine transformed). Significant terms and p-values are shown in bold[1].

Approach	Statistical method	Predictors	F	d.f.	P
Cultures	GLMM	Bird species identity	1.10	16,134	0.103
		Bacterial density	5.29	1,134	**0.023**
ARISA	GLMM	Bird species identity	1.27	16,107	0.219
		OTUs richness	2.37	1,107	0.126
	PA	Bird species identity	1.23	16,46	0.298
		Bacterial assemblage (GOWER)	1.09	62,46	0.070
HiSeq	GLMM	Bird species identity	1.20	16,118	0.282
		OTUs richness	0.11	1,118	0.736
	PA	Bird species identity	1.80	16,15	0.273
		Bacterial assemblage (Unweighted UniFrac)	2.07	104,15	**<0.001**
	PA	Bird species identity	1.03	16,50	0.273
		Bacterial assemblage (Weighted UniFrac)	0.76	69,50	0.488

[1]Bacterial density on eggshells was estimated as the number of colony forming units per cm^2 of mesophilic bacteria and GLMM was applied. OTU richness was estimated as the number of OTUs in ARISA and HiSeq. OTU richness from ARISA approach was log-transformed in order to reach normality. Bacterial assemblages were estimated by means of Automatic Ribosomal Intergenic Spacer Analysis (ARISA) or by means of high-throughput sequencing (Illumina HiSeq platform). For comparison of bacterial assemblages, Pearson similarity index was applied for ARISA data, and Weighted and Unweighted UniFrac distances for HiSeq data and Procrustes ANOVAs (PA) were performed.

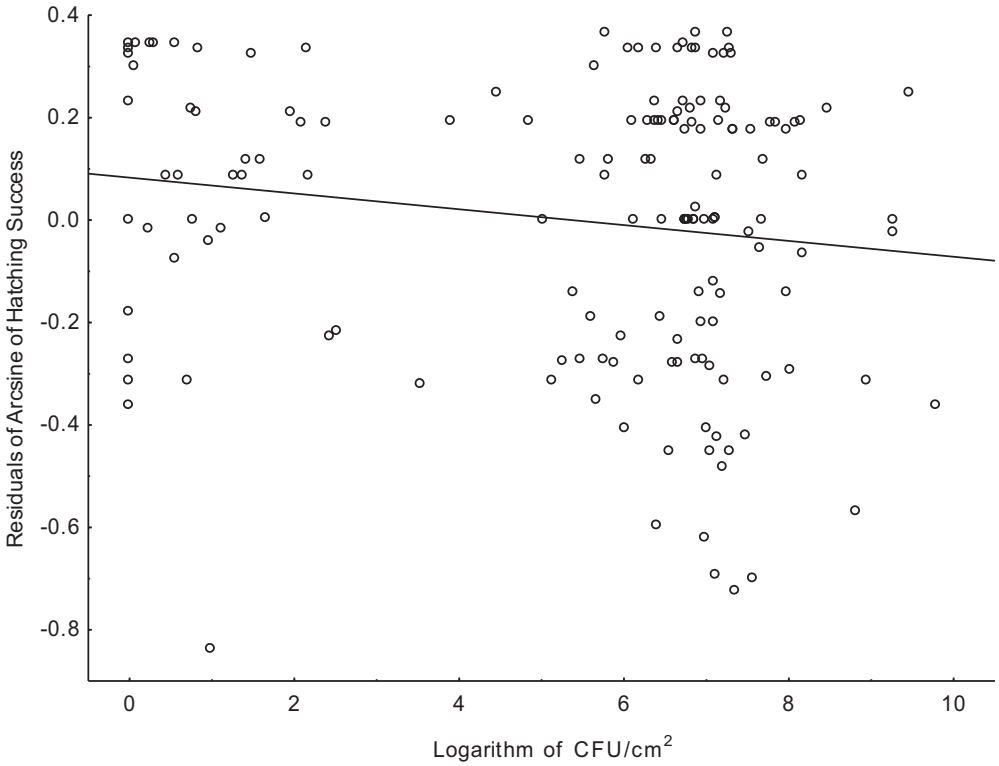

Figure 2. Correlation between hatching success (percentage of eggs that hatch in a clutch) and log-transformed of mesophilic bacterial density on eggshells at the beginning of the incubation after correcting for differences among species. Bacterial density was calculated by number of colony forming units that grew in Tryptic Soy Agar plates divided by egg surface (cfu/cm^2). Line represents the trend line ($R^2 = 0.02$).

ARISA has been used extensively in microbial ecology for characterizing bacterial community, the advantages of next generation sequencing make this technique more recommendable in studies of microbial ecology.

Taxonomical composition of bacterial communities

Few studies have explored the bacterial community on avian eggshells using culture-independent methods (Shawkey *et al.* 2009; Grizard *et al.* 2014; Lee *et al.* 2014; Grizard *et al.* 2015). The bacterial community found on eggshells in the present study resembles those described in previous papers. The bacterial profile present in our samples, rich in Gammaproteobacteria, has

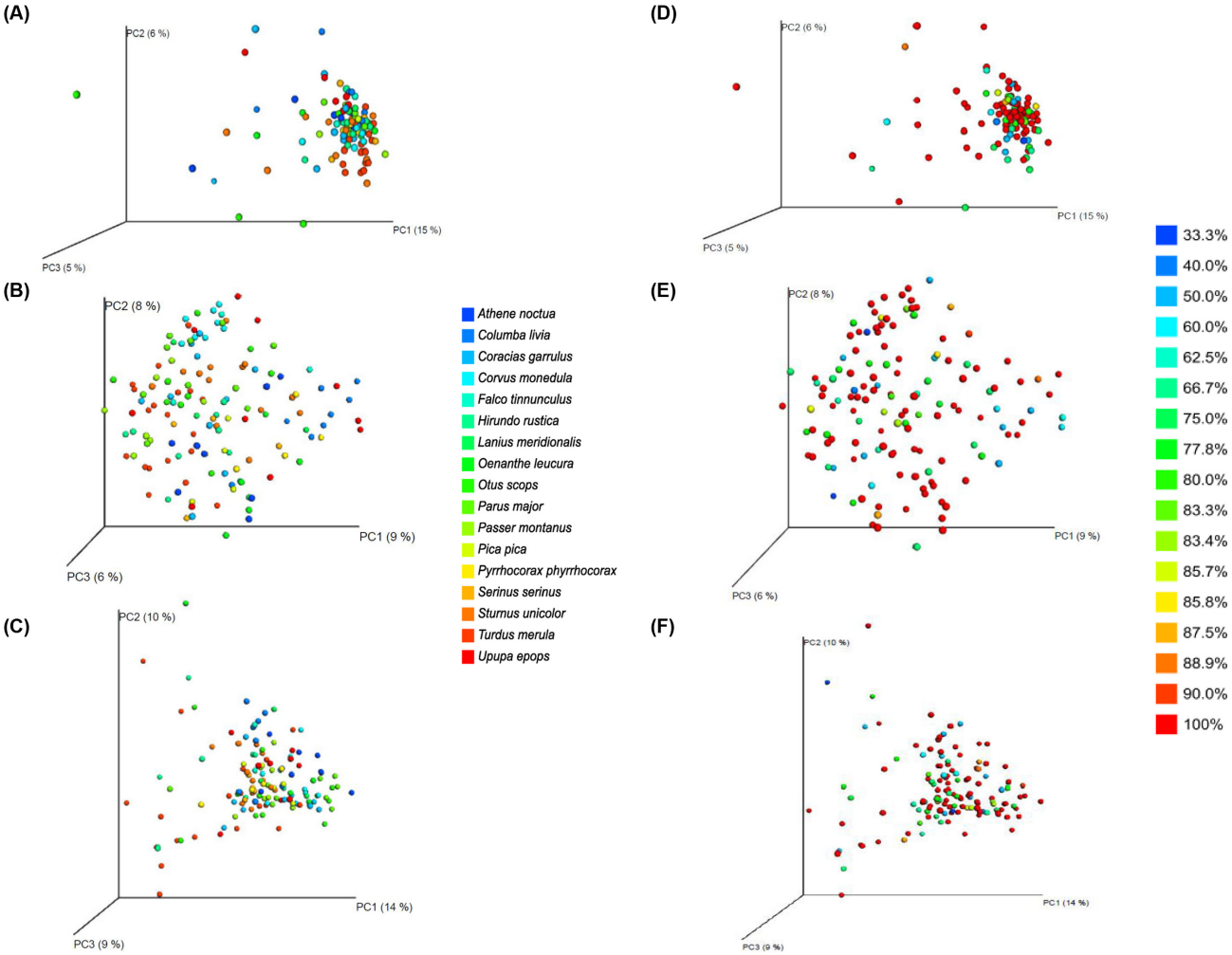

Figure 3. 3D plots showing the first three axes of Principal Coordinate Analyses (PCoA) of bacterial community of bird eggshells. Percentages show the proportion of explained variance for each axis. Each dot represents the bacterial community of the complete clutch in a nest. (**A** and **D**) represent bacterial community estimated by Gower distance of ARISA, (**B** and **E**) by means of Unweighted UniFrac distance, and (**C** and **F**) by means of Weighted UniFrac. (A, B and C) are colored by avian species while (D, E and F) are colored by hatching success gradient.

Table 3. Phylogenetic general least square (pGLS) analyses evaluating the effects of bacterial density or community on bird eggshells on the hatching success in a comparative approach[2].

Approach	Variable/matrix	R^2 adjusted	F	d.f.	P
Cultures	Bacterial density	−0.02	0.76	1,16	0.461
ARISA	Gower distance matrix	0.07	1.07	1,16	0.397
HiSeq	Unweighted UniFrac distance matrix	0.10	1.64	1,16	0.475
	Weighted UniFrac distance matrix	0.05	0.83	1,16	0.583

[2]Bacterial density was estimated as the geometric mean of colony forming units per cm^2 per bird species. Bacterial assemblages for each bird species was calculated as the prevalence of every OTU in each bird species (ratio of an OUT is present in an avian species), for both Automatic Ribosomal Intergenic Spacer Analysis (ARISA) and high-throughput sequencing (Illumina HiSeq platform). Pearson similarity index was applied to ARISA for calculating the distance matrix and Weighted and Unweighted UniFrac distance from HiSeq.

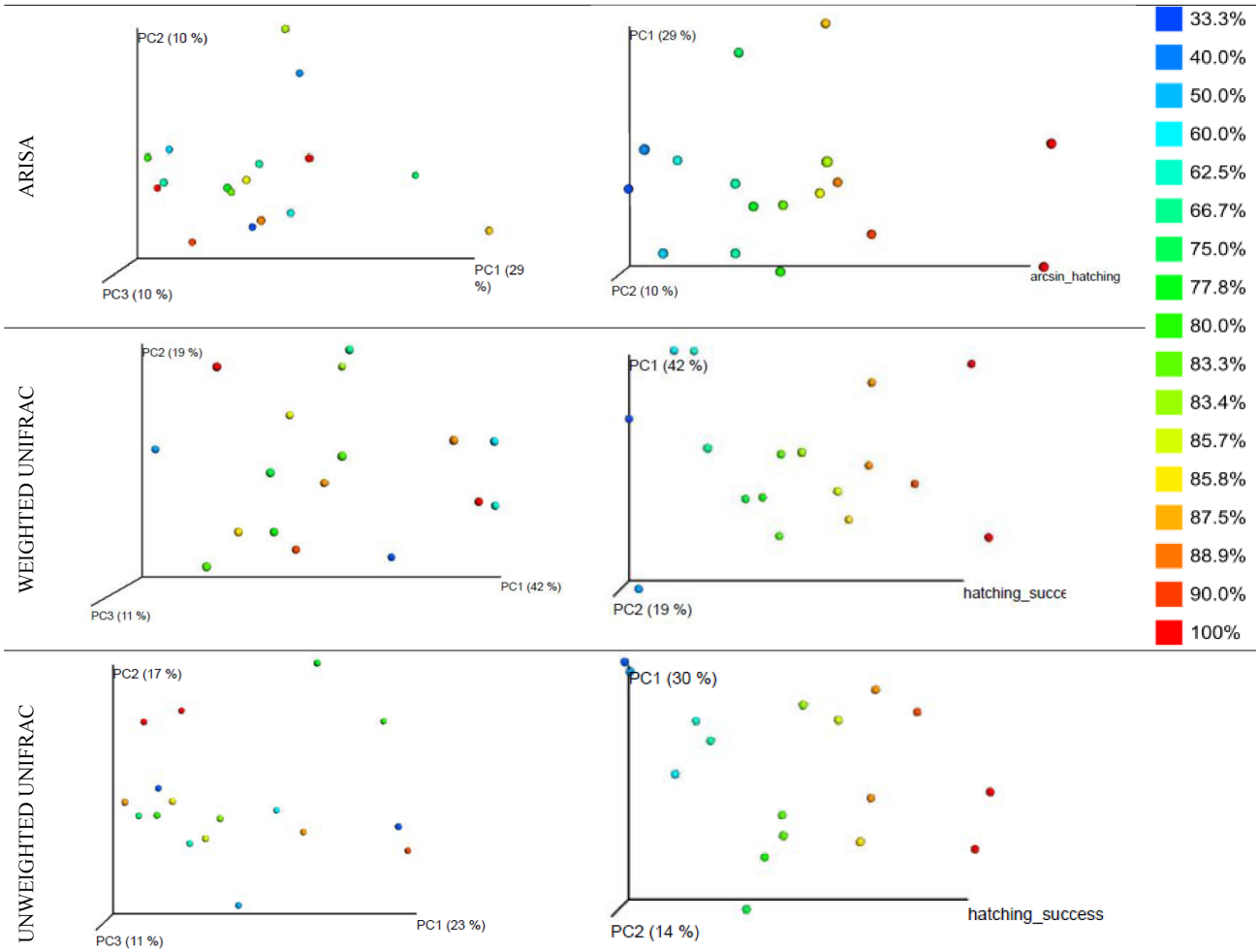

Figure 4. 3D plots showing the first three axes of PCoA of bacterial community on eggshells of different species of birds. Percentages show the proportion of explained variance for each axis. Each dot represents the prevalent bacterial community of each avian species. Each OTU prevalence was calculated as the percentage of presence of that OTU in the nest of each avian species. ARISA bacterial community was estimated by Gower distance , and HiSeq bacteria communitites by Unweighted and Weighted UniFrac distances..

been previously reported in the feral dove, *Columba livia* (Grizard *et al.* 2014), or the magpie *Pica pica* (Lee *et al.* 2014). Moreover, Shawkey *et al.* (2009) found a rich community of Betaproteobacteria in the pearl-eyed thrush *Margarops fuscatus*. The similarities in eggshell bacterial composition between ours and previous studies also apply to lower taxonomic levels. *Pseudomonadaceae* is a common family on eggshells and nests of blue tits, *Cyanistes caeruleus* and great tits, *Parus major* (Goodenough and Stallwood 2010), house wrens Troglodytes aedon (Singleton and Harper 1998) and common magpies (Lee *et al.* 2014). *Pseudomonas*, a saprophytic and opportunistic bacteria (Bergey *et al.* 1984) that is commonly found in eggshells of these species and those analyzed in our study has traditionally been related to infection and disease in birds (Benskin *et al.* 2009). The detected anti-*Pseudomonas* activity of egg contents (Kovacs-Nolan *et al.* 2005) confirms that these bacteria seem to play an important role in the viability of bird eggs. However, we can only speculate that particular strains could be related with hatching failure, and, only in some particular environmental conditions, they are able to infect egg contents. For instance, *Neisseria sp.* is the main strain producing hatching failures in the arctic goose *Anser nivalis* (Hansen *et al.* 2015) suggesting the importance of other bacteria in addition to *Pseudomonas*. In other cases, common and commensal stains are related with egg content infection as *Escherichia coli* and *Enterococcus sp.* (in the saker falcon *Falco cherrug*: Janos *et al.* 2007), or *Escherichia coli and Staphylococcus epidermitis* (in the house sparrow *Passer domesticus*: Pinowski *et al.* 1994). Some of these genera were found in low abundance in the present study, although they were not significantly related with hatching failure.

Intraspecific approach

At the intraspecific level, our findings support the predicted negative relationship between the density of aerobic mesophilic bacteria on the eggshell at the beginning of the incubation and hatching success. This result suggests two possible scenarios. The first one is that bacterial density per se affects hatching success independently of the composition of the bacterial community. The second scenario suggests a link between potentially harmful bacteria that were present on the eggshells at the time of sampling (i.e. the start of the incubation) and bacterial density

and probability of embryo death. In this sense, we detect a significant association between eggshell bacterial assemblage and hatching success, but only when using the Unweighted UniFrac metric of bacterial community. Both Weighted and Unweighted UniFrac distance matrixes were calculated because we do not know a priori the relative importance of rare bacterial taxa. Weighted UniFrac gives more weight to the most abundant bacterial taxa, while Unweighted UniFrac gives similar weight to all bacterial species present (Lozupone *et al.* 2007). The fact that only Unweighted UniFrac was significantly related to hatching success points out the importance of minority and rare bacterial taxa on the embryos' viability, although we did not find a significant correlation between prevalence of any individual OTU and hatching success. Supporting these speculations, bacterial density and community assemblage were related and, thus, it is still possible that the negative association between hatching success and bacterial density for mesophilic bacteria is due to the presence of certain bacterial groups on the eggshells, the consortium of some rare bacterial species or maybe facilitating others to pass through the eggshell even though they are not harmful by themselves. It is worth to mention here that for characterizing bacterial communities we used richness values and OTU assemblages as a whole (i.e. distance matrixes). We just speculate that only a few bacterial strains are able to penetrate the eggshells (Cook *et al.* 2005a; Wang, Firestone and Beissinger 2011), which may be at lower density on eggshells but their presence could be related to bacterial abundance. This idea is really attractive, but empirical evidence and experimental studies are necessary for disentangling these relationships.

Birds by means of early incubation are able to control bacterial growth on the eggshell (Cook *et al.* 2005a; Shawkey *et al.* 2009) and induce changes in their bacterial communities (Shawkey *et al.* 2009; Potter *et al.* 2013; Brandl *et al.* 2014; Grizard *et al.* 2014; Lee *et al.* 2014) that favor proliferation of less harmful bacterial strains (Cook *et al.* 2005a; Brandl *et al.* 2014; Lee *et al.* 2014), thereby reducing probability of hatching failures (Cook *et al.* 2003, 2005b). These beneficial effects of incubation are likely mediated by the associated drying effect of the incubation (D'Alba, Oborn and Shawkey 2010; Ruiz-de-Castañeda *et al.* 2011c), particularly at the beginning of the incubation period, when most of the trans-shell bacterial infections occur (Cook *et al.* 2005a, 2005b; Shawkey *et al.* 2009, but see Wang, Firestone and Beissinger 2011). Trying to reduce the effect of incubation on bacterial communities of eggshells, eggs were sampled after the laying stage in our study. However, some of the species investigated start to incubate before the end of egg laying, and these species tend to harbor more bacteria on their eggshells than those starting to incubate with the last eggs (Peralta-Sánchez *et al.* 2012). Thus, because these species could also vary in hatching success, interspecific variation in incubation behavior could explain the detected intraspecific association between bacteria and hatching success. This possibility is, however, unlikely; first because the detected positive association between bacterial density and hatching success was corrected for the random effect of species identity and, second, because the slope of the association between hatching success and bacterial density was similar in different species. Our results are in any case correlative, and experiments would be necessary for establishing further cause-effect relationships.

Interspecific approach

Associations between bacterial density and hatching success have been detected in poultry (Board and Fuller 1994), for one tropical bird species, *M. fuscatus* (Cook *et al.* 2005b), and in a comparative study of European birds (Soler *et al.* 2012). In the present study, we found that species with the highest eggshell bacterial densities were not those with the lowest rates of hatching success. Moreover, richness and phylogenetic structure of bacterial communities of different species was not significantly related to hatching success. These results may suggest that species experiencing a high risk of bacterial infections (i.e. eggshell bacterial densities) are also those laying eggs with more effective defenses against trans-shell bacterial infection (Soler *et al.* 2011a), breaking up the expected association. Interspecific variation in number and size of eggshell pores and thickness (Becking 1975; Ar, Rahn and Paganelli 1979; Mallory and Weatherhead 1990; Zimmermann and Hipfner 2007), in diversity and quantity of antibacterial proteins on the eggshells (Panheleux *et al.* 1999), cuticle (Wellman-Labadie, Picman and Hincke 2007, 2008a), and albumen of the eggs (Wellman-Labadie, Picman and Hincke 2007; Shawkey *et al.* 2008; Wellman-Labadie, Picman and Hincke 2008b; Hirose *et al.* 2011), and in diversity and concentration of maternal antibodies in the egg yolk (Hasselquist and Nilsson 2009) could affect probability of embryo infection (Board and Fuller 1994). We have not considered these antimicrobial defenses that have to be adjusted to the risk of bacterial infections related to environmental conditions (Cook *et al.* 2003; Cook *et al.* 2005a; Soler *et al.* 2011b; Wang, Firestone and Beissinger 2011), and that might explain the absence of an interspecific correlation between bacterial assemblage and hatching success. Previous evidence supporting such a relationship in Soler *et al.* (2012) did not use hatching success of the nests that were sampled for bacterial density on the eggshell, but collected data from the literature instead. Soler *et al.* (2012) also used a larger list of species sampled in Spain and Denmark. However, other scenarios are also possible. For instance, (i) the subset of species included in our study is not completely representative of bacteria–bird relationships (i.e. only includes altricial species and mainly nesting above ground); (ii) this effect is not that strong in relatively dry environments like the south of Spain (in comparison with tropical areas) (Wang, Firestone and Beissinger 2011); (iii) or this association is driven by other components of bacterial composition diversity (i.e. functional diversity) or interspecific interactions within the community (for the same Unifrac or richness different effects may be found depending on the antagonistic/mutualistic interactions of certain bacteria).

Final remarks

Even though we failed to detect a negative association between bacterial density on eggshells and hatching success across 17 avian species, we found the predicted association at the intraspecific level, which supports the extended idea that bacteria are important selective agents for wild bird eggs (Deeming 2002; Cook *et al.* 2005a, 2005b). Similarly, we found some evidences of the effect of bacterial community composition (Unweighted UniFrac) on hatching success within species, indicating the potential importance of rare bacterial groups in this context. Our findings support the importance of the Rare Biosphere (Pedrós-Alió 2012) as well as open new avenues to understand their role in bird fitness.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of interest. None declare.

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